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DNA ISOLATION OF MICROBIAL CONTAMINANTS IN AVIATION TURBINE FUEL VIA TRADITIONAL POLYMERASE CHAIN REACTION (PCR) AND DIRECT PCR

Preliminary Results

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Microbially contaminated aviation fuel cause the Air Force increased maintenance and replacement costs from problems such as fuel gauge malfunctions, fuel line and filter plugging, and corrosion. As a result, there is considerable interest in identifying microbial growth and finding strategies to mitigate it. Previous research to isolate and identify aviation fuel microbial contaminants has used cultivation-based methodologies. This study aimed to investigate newer molecular methods to more comprehensively characterize the bioburden in aviation fuel supplies. Several fuel samples were analyzed for bacterial contamination using two distinct methods: a cultivation-independent method (direct PCR) and a traditional cultivation-dependant method. A total of 36 bacterial genera were identified, including 28 genera which have not been previously reported in aviation fuel. Nearly 62% of the new bacterial genera were isolated with the cultivation-independent method only, 33% with both methods, and only 5% with the cultivation-dependant method only.

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ABSTRACT

Microbial contaminated aviation fuel causes the Air Force increased maintenance and replacement costs from problems such as fuel gauge malfunctions, fuel line and filter plugging, and corrosion. As a result, there is considerable interest in identifying microbial growth and finding strategies to mitigate it. Previous research to isolate and identify aviation fuel microbial contaminants has used cultivation-based methodologies. This study aimed to investigate newer molecular methods to more comprehensively characterize the bioburden in aviation fuel supplies. Several fuel samples were analyzed for bacterial contamination using two distinct methods: a cultivation-independent method (direct PCR) and a traditional cultivation-dependent method. A total of 36 bacterial genera were identified, including 28 genera which have not been reported in aviation fuel. Nearly 62% of the new bacterial genera were isolated with the cultivation-independent method only, 33% with both methods, and only 5% with the cultivation-dependent method only. The cultivation-independent method identified an average of four times the amount of contaminants as identified using the cultivation-dependent method. The newer method, although in need of refinement, demonstrates great promise to more completely characterize the aviation fuel bioburden. As the microbial contaminants are identified, significant work will be dedicated to studying the molecular dynamics and behavior of the contaminant communities to create a specifically targeted solution to prevent aviation fuel microbial contamination.

PREFACE

The purpose of this study was to investigate newer molecular methods to more completely identify bacterial contaminants in microbially contaminated aviation fuel systems. The methodologies explored here take advantage of some of the latest advances in microbiology. The results presented in this report are the preliminary findings to a study currently being conducted by the Air Force Research Laboratory, Propulsion Directorate, and Fuels Branch. The authors thank Dr. Michelle Rauch and Dr. Charles Bleckmann for their technical contributions to this study, including their role in laying the groundwork for the current effort, and their continuing support of the study with their technical advice. The results of this completed study will equip the Fuels Branch with essential information to engage in research to understand the molecular dynamics and behavior of the common aviation fuel microbial contaminants when in their natural environment. It is vital to answer the basic questions of what microorganisms currently exist, their frequency, and which ones contribute most significantly to the formation of biofilms and other types of aircraft system contamination before a specifically targeted and reliable solution can be developed.

1. Summary

Previous research to isolate and identify aviation fuel microbial contaminants has used cultivation-based methodologies. The goal of this study was to investigate a newer and more efficient method to comprehensively characterize the bioburden in aviation fuel supplies. Several fuel samples were analyzed in this study using two distinct methods: a cultivation-independent method (direct PCR) and a traditional cultivation-dependent method. The cultivation-independent method identified an average of four times the amount of contaminants as identified using the cultivation-dependent method. The newer method demonstrates great promise to more completely characterize the aviation fuel bioburden.

2. Introduction

Microbial contamination of military aviation fuel has been recognized as a significant issue since the 1950's (1,2,3,4). Problems caused by microbial contamination include the maintenance, replacement, and mission failure costs arising from fuel gauge malfunctions, fuel line and filter plugging, and corrosion (5). These operational problems led to a joint service research endeavor, of which the United States Air Force (USAF) was a crucial member, to more fully understand the problem and to develop a viable solution (3). An unexpected fix to the problem arose out of an unrelated research study to prevent fuel icing at low temperatures. The fuel system icing inhibitor (FSII), ethylene glycol monomethyl ether (EGME), that the USAF introduced into JP-4 fuel in the early 1960's, was found to have biocidal activity (3). The new FSII additive, combined with some improved housekeeping procedures to keep water out of the fuel, resulted in a steady decline of microbial contamination problems (6). As a result, the joint research study was brought to a close and the issue was considered resolved. In the early 1980's the USAF replaced EGME with a less toxic chemical which had been used in Navy JP-5 fuel, di-ethylene glycol monomethyl ether (DiEGME) (4,5,7,8). Shortly after this change the USAF transferred from the use of the volatile fuel, JP-4, to a higher molecular weight kerosene based fuel, JP-8. These two factors may have led to the steady increase of microbiological contamination of USAF aviation fuel systems observed in the last ten years (5).

Several recent operational problems occurring between 2000 and 2002 led to a joint research effort by the Air Force Research Laboratory at Wright-Patterson AFB, OH, Propulsion Directorate, Fuels Branch and the Air Force Institute of Technology, Systems and Engineering Department to investigate the issue (5). The research included the isolation and identification of microbial contaminants in USAF aviation fuel samples collected from a wide array of airframes covering a diverse geographical range of operational Air Force bases. This study resulted in the isolation of many additional microbial contaminants not previously found in aviation fuel supplies. Several factors could have contributed to this result including, but not limited to, a change in the microbial consortia and an improvement of laboratory techniques (5).

As summarized and reported in Rauch et al. (2005), Table 1 displays the historical fuel microbial contamination research ranging from the first study conducted in the late 1950's to the most recent study completed in 2003.

The aforementioned studies all used traditional methods based on cultivation to isolate and identify the microbial contaminants in each sample. However, cultivation methods, regardless of their accepted use in the past, have several limitations. Research conducted in the last 10-20 years has revealed the existence of many viable but unculturable species (15,16). In fact, literature suggests that only 1% of environmental microbes, the microbes that habitually thrive in jet fuel systems, have actually been identified and cultivated (17). In Table 2, Amann et al. (1995) summarized the culturability of several types of environmental microbial communities (15). The tiny percentage of culturable environmental microbes demonstrates significant limitations for microbial isolation and identification methodologies that are dependent on cultivation.

To complicate the issue further, most environmental samples house a diverse community of microorganisms where there exist many symbiotic relationships (18). To isolate and study a single contaminant would be a futile effort since the molecular dynamics, activity, and behavior of that particular microbe may be very different when in its community rather than in an isolated culture (18).

Table 1. Microbial Contaminants Isolated from Commercial and Military Aviation Fuel 1958-Present as Summarized and Reported by Rauch et al. (2005)

Bacteria	JP-4 1958 - 1966[1,3,6,9]	Jet A 1988 - 1997[10,11]	Jet A-1 1998 & 1999 [12,13]	JP-8 2002 [14]	JP-8 2003[5]
Acinetobacter (calcoaceticus, cerificans)		Yes	Yes		
Arthrobacter			Yes		Yes
Aerobacter aerogenes	Yes	Yes	Yes		
Aeromonas sp.		Yes	Yes		
Alcaligenes		Yes	Yes		Yes
Brevibacterium ammoniagenes	Yes		Yes		
Desulfovibrio sp. (SRB)	Yes	Yes	Yes		
Dietzia sp.					Yes
Escherichia sp.	Yes				
Enterobacter (cloacae, glomerans)			Yes		
Flavobacterium (arborescens, diffusum)	Yes	Yes	Yes		
Kocuria rhizophilia					Yes
Leucobacter komagatae					Yes
Micrococcus sp.	Yes	Yes	Yes		Yes
Pantoea ananatis					Yes
Streptomyces sp.			Yes		
Staphylococcus sp.					Yes
Sphingomonas sp..					Yes
Serratia (marcescens, odorifera)			Yes		
Bacillus sp. (acidocaldarius, + others*)*	Yes	Yes	Yes	Yes	Yes
Pseudomonas sp. (aeruginosa, + others*)*	Yes	Yes	Yes		
Fungi					
Acremonium sp. (strictum)		Yes	Yes		
Aspergillus sp. (niger, fumigatus, + others*)*	Yes	Yes	Yes		
Aureobasidium pullulans	Yes		Yes		Yes
Candida sp. (famata, lipolytica + others*)*		Yes	Yes		
Discophaerina fagi					Yes
Exophiala jeanselmei				Yes	
Fusarium sp. (moniliforme, + others*)*		Yes	Yes		
Hormoconis (Cladosporium) resinae	Yes	Yes	Yes	Yes	
Helminthosporium sp.	Yes		Yes		
Paecilomyces (variotii + others*)*	Yes	Yes	Yes		
Penicillium sp. (corylophilum, + others*)*	Yes	Yes	Yes		
Phialophora sp.		Yes	Yes		
Rhinocladiella sp.			Yes		
Rhodotorula sp.		Yes	Yes		
Trichosporium sp.			Yes		
Trichoderma sp. (viride + others*)*		Yes	Yes		

Table 2. Culturability Determined as a Percentage of Culturable Bacteria in Comparison with Total Cell Counts as Summarized by Amann et al (1995).

Habitat	Culturability (%)[a]
Seawater	0.001-0.1
Freshwater	0.25
Mesotrophic lake	0.1-1
Unpolluted estuarine waters	0.1-3
Activated sludge	1-15
Sediments	0.25
Soil	0.3

[a] Culturable bacteria are measured as CFU.

In the last decade, recent advances in molecular methods and analysis have contributed to the development of many new microbial isolation and identification methods (19,20,21). In particular, developments in the molecular analysis of rRNA have made it possible to isolate a diversity of environmental contaminants without the need for cultivation (19,20). These new methods allow researchers to capture an unbiased representation of microbial contaminants in both complex and simple environmental samples (19,20). The first attempts to use rRNA to characterize an environmental sample were conducted nearly 20 years ago (15,20). Since then, significant refinement of the methodology has occurred (20). The 16S and 18S rRNA partial gene in prokaryotes and eukaryotes, respectively, are optimal for microbial identification because they provide both genetic specificity and sensitivity (20,22). Within the 16S bacterial rRNA gene there are domains of high conservation that act as a general template to identify contamination (20). In addition, there are also domains of high specificity that allow for microbe identification as detailed as the genus and species level (20). Not only is direct microbial isolation possible with this technology, but techniques such as in-situ hybridization makes it possible to quantitatively identify specific contaminants very quickly without disturbing the natural environment (17). Many more methods continue to be developed and refined as the knowledge of the field expands (17,21). Some additional literature encountered in the course of this study appears in a bibliography at the end of this paper, and is not specifically referenced in the text.

The purpose of the work presented in this report was to utilize these newer molecular methods to more comprehensively characterize the microbial contamination that exists in aviation fuel supplies. The results presented are preliminary findings from an ongoing study to fully characterize the microbial contamination burden on USAF and civilian aviation fuels. This report provides insight into the use of non-cultivation methods to identify microbial contaminants and provides a comparison of these newer methods with traditional cultivation-based methods. The outcome of the completed study, a comprehensive characterization of aviation fuel microbial contamination, is just the first step towards a safe, successful, and permanent resolution to this issue.

Once the field study analysis is complete and a complete list of microbial contaminants has been compiled, laboratory analysis of the activity and behavior of aviation fuel microbial communities can be pursued. This analysis is the essential precursor to developing a reasonable solution to prevent harmful microbial contamination from all aviation fuel systems.

3. Methods, Assumptions, and Procedures

3.1 Sample Collection

Several civilian aircraft in long term storage were sampled in December of 2004, January of 2005, and again in April of 2005; the results of which are presented in this report. These aircraft were from commercial airbases in Victorville, California and Roswell, New Mexico. There were no clear differences in bacteria identified from planes in each location, so the results from these planes have been combined. In June 2005, sampling of active military heavy aircraft began, however, these results are still being processed and will be presented in the final report when the study is complete. The sample collector drained fuel/water from the low point sumps in each wing and center body tank into HDPE 1L wide-mouth containers (Environmental Sampling Supply, Oakland, CA). Container preparation by the manufacturer included a non-phosphate detergent wash, multiple tap water and ASTM Type I de-ionized water rinses, 1:1 HNO_3 rinses, and oven drying. Two liters were collected from each sump and labeled with aircraft and tank identifiers. The sampling implements were sterilized with a 10% bleach solution and rinsed 3 times with sterile water between aircraft sampling. The first liter of sample was shipped to AFRL/PTRG by overnight air and was available for laboratory testing within 24 hours of sampling. The second liter of fuel/water was retained at the flight line for immediate analysis using a commercial adenosine triphosphate (ATP) test kit (Hy-LiTE®, Merck KGaA, 64271 Darnstadt, Germany). All fuel samples from the first fuel/water liter were tested using cultivation-based commercial test kits after laboratory analysis was complete (described below). Samples were tested for fungi using a commercial FUELSTAT™ *resinae* kit (Conidia Bioscience, UK), for bacterial colony forming units (CFU) using MicrobeMonitor ^{2} ™ test kits (ECHA Microbiology Limited, UK), and for general fungal/bacterial activity using ATP test kits (Hy-LiTE®).

3.2 Microbial Extraction from Fuel Samples (Laboratory Analysis of First Liter)

A mixed aliquot was selected for analysis from all samples. If samples contained more than 1 mL aqueous phase, both the fuel phase and aqueous phase were selected for analysis in addition to the mixed aliquot. To prepare the mixed aliquot, samples were shaken by hand for a minimum of 30 seconds prior to sampling. 60 mL mixed fuel was collected in a sterile, disposable 60 mL syringe (Becton Dickinson and Company, Franklin Lakes, NJ). A sterile, hydrophobic 0.45 µm, 26 mm diameter, luer-lock tip filter (Corning, Corning, NY) was attached to the tip of the syringe and the fuel was filtered. The filter was removed from the syringe and placed in a laminar flow hood to dry. A new sterile 60 mL syringe was used to collect 60 mL sterile air. The filter was attached to the tip of the syringe and the air passed through the filter. This was repeated several times until the filter paper was dry. The filter was attached to the tip of a new syringe and 0.5 mL sterile water was collected through the filter into the syringe. The filter was removed and the water placed into a sterile 1.5 mL eppendorf tube. The filter was again attached to the tip of the same syringe and 0.7 mL sterile water was collected through the filter into the syringe. The filter was removed and the contents in the syringe placed into a new, sterile 1.5 mL eppendorf tube.

The identical procedure as described above was conducted to analyze the fuel phase. For aqueous phase analysis, 2 mL collected from the aqueous phase was placed into a 2 mL eppendorf tube. The sample was centrifuged in an Eppendorf Centrifuge 5810 (Fisher Scientific, Fair Lawn, NJ) for 4 minutes at 13,000 rpm and the supernatant decanted and discarded. Two milliliters of sterile water was added to the pellet, the contents were mixed gently by inverting the tube several times, and then centrifuged for four minutes at 13,000 rpm. The supernatant was discarded and the same procedure was repeated at least once. One milliliter of sterile water was added to the final pellet and the contents mixed gently by inverting the tube.

All samples at this point were analyzed using two distinct procedures; microbial cultivation and direct rRNA sequencing. Both techniques provided qualitative information of the bacterial population in each sample.

3.3 Microbial Cultivation and DNA Sequencing

All tubes were cultured and sequenced using an identical procedure to that previously reported (5) with exception of the specific details discussed below. Samples were spread onto three types of agar: SDA and TSBA agar for aerobes, and BHI agar for anaerobes. The cell cultures were incubated at 29° C for aerobes and 35° C for anaerobes. No dilutions were made prior to plating. Aerobes typically grew in one to four days, and anaerobes typically grew in two to six days, but sometimes as quickly as one. An individual colony was harvested for each bacterial type grown, and this colony was restreaked on a new plate of the same agar. A colony from the fourth quadrant was then used for PCR. Before lysing cells and performing PCR, several dilutions were made of the neat cell suspension to fully optimize the amount of DNA recovered from each sample. After analysis by agarose gel electrophoresis, the dilution from each sample with the most effective 16S 500bp amplification was selected for further work. PCR amplimers were cloned as described previously (5). Purification of plasmid DNA and the DNA sequencing reactions were performed by MWG Biotechnology Sequencing Laboratory (MWG-Biotech, High Point, NC). DNA sequencing was accomplished using M13 forward and reverse primers.

3.4 Direct rRNA Extraction and DNA Sequencing

All tubes analyzed by the protocol described above were also analyzed using a direct rRNA extraction method to eliminate microbial cultivation as a part of the procedure. 100 µL of sample was added to a 0.2 mL microtube and several dilutions were made as detailed above. All dilutions were heated at 99°C for 10 minutes and PCR was performed as described previously (5). After analysis by gel electrophoresis, the dilution with the most successful amplification was selected for further work. PCR amplimers were cloned identically as described previously (5). Purification of plasmid DNA from 48 clones per plate and the DNA sequencing reactions were performed by MWG Biotechnology Sequencing Laboratory (MWG-Biotech, High Point, NC). DNA sequencing was accomplished using M13 forward and reverse primers.

4. Results and Discussion

As stated earlier in this report, a significant amount of research has been conducted since the late 1950's on aviation fuel microbial contamination (Table 1) (5). All the microbial species previously found in aviation fuel systems were isolated and identified through the use of cultivation-based methods. The current research focused on a method that avoided any type of cultivation and directly compared those results with a cultivation-based method nearly identical to that used by Rauch et al (2005). The microbial contaminants identified in this study have been added to those in Table 1 and the results are shown in Table 3. Microorganisms in white were found in previous studies. Other microorganisms shown have not been reported in aviation fuel prior to this study.

The current study identified a total of 36 microbial genera, including 28 genera (72.4%) that were found in jet fuel for the first time according to the available literature (1,3,5,6,9,10,11,12,13,14). The staggering increase in types of bacteria isolated from aviation fuel since 1958 is illustrated in Figure 1.

The primary factor that distinguishes the current study from previous studies is the use of a direct PCR cultivation-independent method. As stated earlier in this report, microbial cultivation has many limitations for contaminant identification and isolation in a microbially diverse sample (as shown in Table 2). Therefore, the experimental research conducted in this study involved both a microbial cultivation inclusive method and a microbial cultivation exclusive method (direct PCR) for the purposes of comparison. The expected result was a significantly greater rate of contaminant isolation and identification using a cultivation-independent method. Figure 2 compares each of the 10 fuel samples analyzed using both methods. On average, the cultivation-independent method identified nearly four times as many microbial contaminants as the cultivation-dependent method.

Table 3. Aviation Fuel Microbial Contaminants Identified from 1958-2005 Including Results from Current Study. Previous Research Summarized by Rauch et. al (2005)

Microbial Contaminants Identified in Aviation Fuel	JP-4 (1958 - 1966)[18,19,20, 21]	Jet A (1988 - 1997)[22, 23]	Jet A (1998 - 1999)[24, 25]	JP-8 (2002)[26]	*JP-8* PRTG study (2003)[27]	Jet A (current study)
Bacteria:						
Acidovorax sp.						Yes
Acinetobacter (calcoaceticus, cerificans)		Yes	Yes			
Arthrobacter			Yes		Yes	Yes
Aerobacter aerogenes	Yes	Yes	Yes			
Aeromonas sp.		Yes	Yes			
Alcaligenes sp. (pardoxus, xylosonxidans)		Yes	Yes		Yes	Yes
Aquabacterium sp.						Yes
Aquasprillum metamorphum						Yes
Bacillus sp. (acidocaldarius, + others)	Yes	Yes	Yes	Yes	Yes	Yes
Bradyrhizobium sp.						Yes
Brevibacterium ammoniagenes	Yes		Yes			
Burkholderia sp.						Yes
Caulobacter subvibroides						Yes
Clostridium sardiniense						Yes
Curtobacterium sp.						Yes
Desulfovibrio sp. (S.R.B.)	Yes	Yes	Yes			
Diaphorobacter nitroreducens						Yes
Dietzia sp.					Yes	
Escherichia sp.	Yes					Yes
Enterobacter (cloacae, glomerans)			Yes			
Ewingella americana						Yes
Flavobacterium (arborescens, diffusum)	Yes	Yes	Yes			
Granulicatella sp.						Yes
Haemophilus parainfluenza						Yes
Herbaspirillum frisingense						Yes
Kocuria rhizophilia					Yes	
Lactococcus lactis						Yes
Leucobacter komagatae					Yes	
Methylobacterium sp.						Yes
Microbacterium (oleovorans)						Yes
Micrococcus sp.	Yes	Yes	Yes		Yes	
Mycobacterium mucogenicum						Yes
Pandoraea sp.						Yes
Pantoea ananatis					Yes	
Photorhabdus luminescens						Yes
Phyllobacterium myrsinacearum						Yes
Propionibacterium acnes						Yes
Pseudomonas (aeruginosa, gladioli, + others)	Yes	Yes	Yes			Yes

Table 3 . Aviation Fuel Microbial Contaminants Identified from 1958-2005, continued

Microbial Contaminants Identified in Aviation Fuel	JP-4 (1958 - 1966)[18,19,20, 21]	Jet A (1988 - 1997)[22, 23]	Jet A (1998 - 1999)[24, 25]	JP-8 (2002)[26]	JP-8 PRTG study (2003)[27]	Jet A (current study)
Rahnella (aquatilis)						Yes
Ralstonia sp.						Yes
Rhiziobium sp.						Yes
Rhodococcus (equi, opacus)						Yes
Rothia (amarae, mucilaginosa)						Yes
Serratia (marcescens, odorifera)					Yes	
Streptomyces sp.			Yes			
Staphylococcus sp.					Yes	Yes
Sphingomonas sp..					Yes	Yes
Streptococcus sp.						Yes
Wolinella succinogenes						Yes

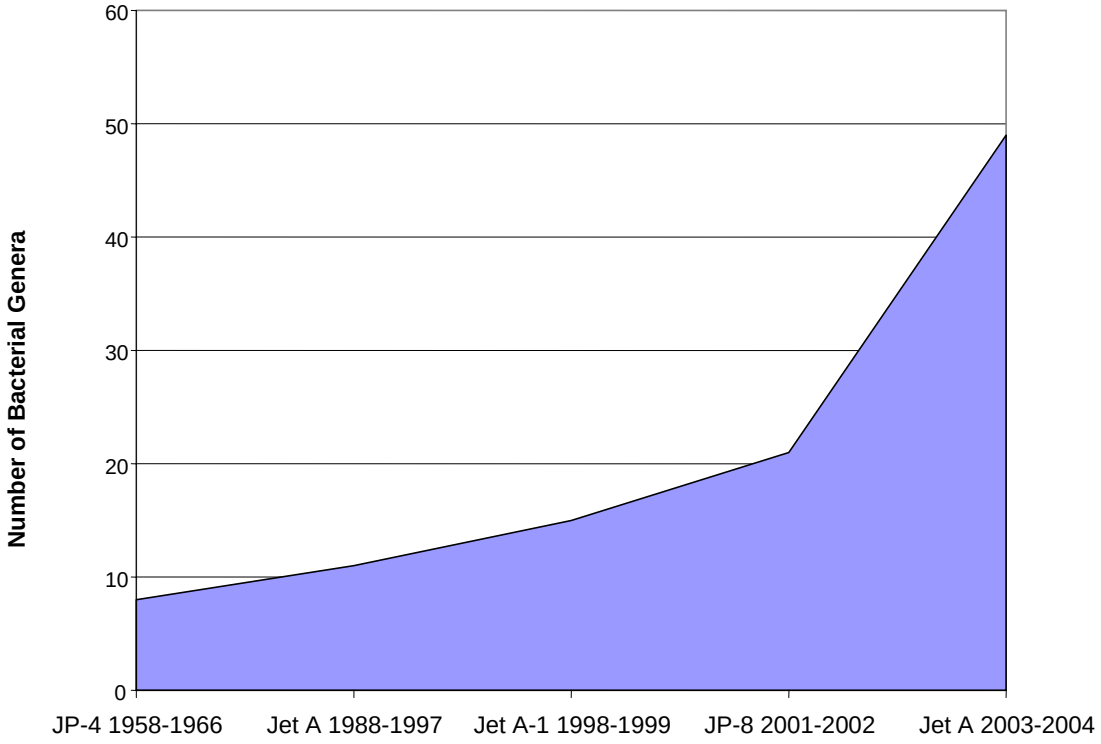

Figure 1. Microbial Contaminants Isolated from Commercial and Military Aviation Fuel 1958-2005.

Table 4 compares the two methods by presenting the data for each microbial species that was isolated and identified. All other data presented in this report are based on microbial genera whereas this extensive list includes all species identified. For any species that contains the word "incomplete" in the direct PCR column, the data for the method has not yet been compiled. Therefore, these few microbes for which not all the data has been gathered are left out of any comparison between the two methods.

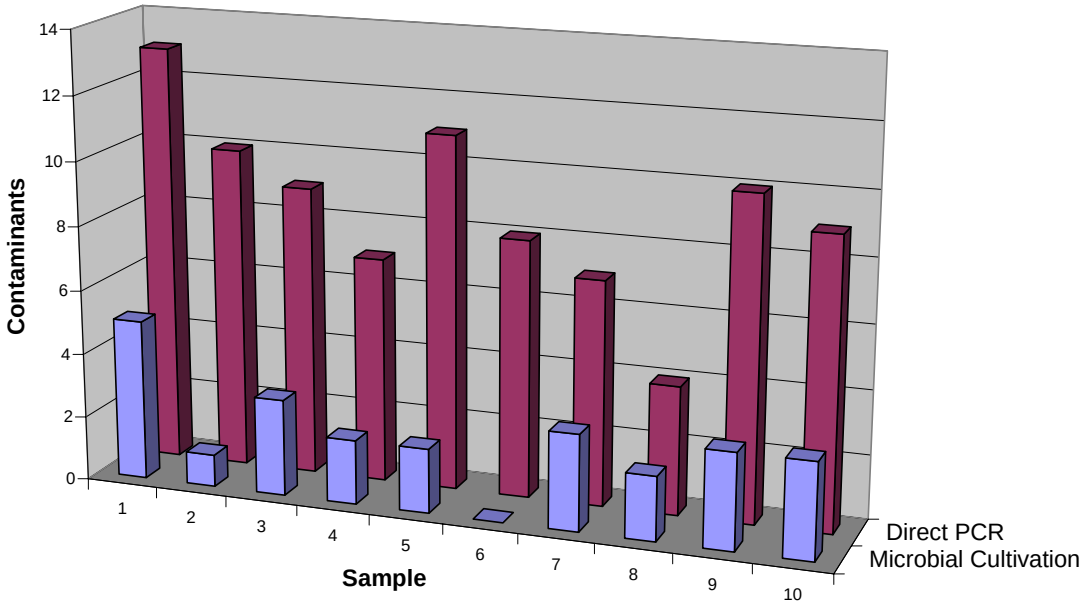

Figure 2. Microbial Contaminants Isolated from Each Sample: a Comparison of the Cultivation-Independent Direct PCR Method and Cultivation-Dependent Method.

Figure 3 provides a comparison of the overall data between the two methods. The pie chart on the left encompasses the total number of bacteria identified in the current study for which complete data have been gathered using both methods. Of the 36 bacteria found in the study, 29 have been tested by both methods. Of those 29, 21 have not been found in aviation fuel samples according to the literature (1,3,5,6,9,10,11,12,13,14). The pie chart on the right depicts the method(s) that were successful in identifying the 21 new bacteria. Nearly 62% of the new bacteria were identified by the cultivation-independent direct PCR method but not by the cultivation-dependent method. Approximately 33% were identified using both methods and only 5% were identified using the cultivation-dependent method only. The identification of bacteria using only the cultivation-dependent method, although only 5%, suggests necessary refinement of the direct PCR method used for this study.

Table 4. Bacterial Species Identified in Current Study by AFRL/PRTG, 2004-2005.

Microbial Contaminants Identified in Aviation Fuel	Isolated with Microbial Cultivation Method	Isolated with Direct PCR Method	Isolation Frequency
Bacteria:			
Acidovorax sp.	No	Yes	4
Acinetobacter iwoffi	No	Yes	1
Alcaligenes paradoxus	No	Yes	2
Alcaligenes xylosonxidans	No	Yes	1
Aquabacterium sp.	No	Yes	1
Aquasprillum metamorphum	No	Yes	4
Bacillus sp.	No	Yes	5
Bacillus licheniformis	Yes	Yes	7
Bacillus anthracis	Yes	Yes	3
Bacillus cereus	Yes	Yes	7
Bacillus thuringiensis	Yes	No	1
Blastobacter sp.	No	Yes	1
Bradyrhizobium sp.	No	Yes	1
Bradyrhizobium elkanii	No	Yes	1
Brevundimonas subvibroides	No	Yes	2
Burkholderia sp.	Yes	Yes	8
Burkholderia cepacia	Yes	No	1
Burkholderia xenovorans	No	Yes	1
Burkholderia glathei	No	Yes	1
Caulobacteraceae bacterium	No	Yes	5
Clostridium intestinale	Yes	No	2
Clostridium sardiniense	Yes	Yes	4
Clostridium sporosphaeroides	Yes	No	2
Curtobacterium sp.	Yes	Incomplete	1
Diaphorobacter nitroreducens	No	Yes	2
Escherichia coli	Yes	No	2
Ewingella americana	Yes	Incomplete	1
Granulicatella sp.	No	Yes	1
Haemophilus parainfluenza	No	Yes	1
Herbaspirillum frisingense	Yes	No	1
Lactococcus lactis	No	Yes	1
Methylobacterium sp.	Yes	Yes	12
Microbacterium sp.	Yes	Incomplete	2
Microbacterium oleovorans	Yes	No	1
Mycobacterium sp.	Yes	Incomplete	1
Mycobacterium mucogenicum	No	Yes	2
Pandoraea sp.	Yes	Yes	6
Photorhabdus luminescens	No	Yes	1
Phyllobacterium sp.	No	Yes	1
Phyllobacterium myrsinacearum	No	Yes	1
Propionibacterium acnes	No	No	2
Rahnella sp.	Yes	Yes	2
Rahnella aquatilis	Yes	Yes	2
Ralstonia sp.	No	Yes	1
Rhiziobium sp.	Yes	Yes	3

Table 4 (continued). Bacterial Species Identified in Current Study by AFRL/PRTG, 2004-2005.

Microbial Contaminants Identified in Aviation Fuel	Isolated with Microbial Cultivation Method	Isolated with Direct PCR Method	Isolation Frequency
Rhodococcus sp.	No	Yes	3
Rhodococcus equi	Yes	Yes	5
Rhodococcus opacus	No	Yes	1
Rhodococcus erythropolis	No	Yes	3
Rothia amarae	Yes	Incomplete	1
Rothia mucilaginosa	No	Yes	1
Streptococcus sp.	No	Yes	1
Streptococcus mitis	No	Yes	1
Streptococcus infantis	No	Yes	1
Variovorax sp.	No	Yes	1
Wolinella succinogenes	No	Yes	1

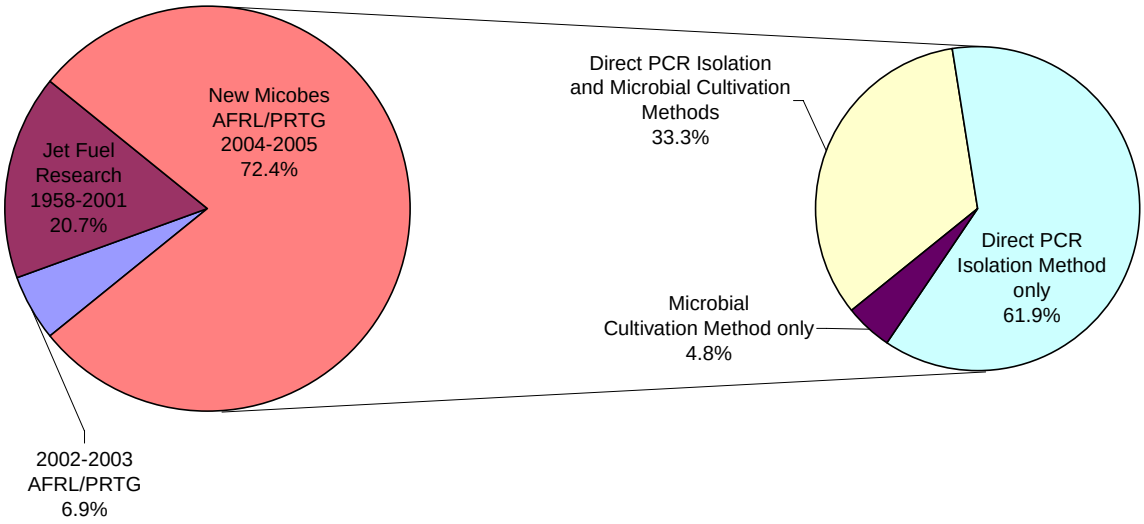

Figure 3. Percentage of Currently Known Microbial Species in Commercial Aviation Fuel Identified, by Chronology (left), and by Isolation Method (right), from AFRL/PRTG 2004-2005

While the results demonstrate the direct PCR method is able to identify more microbial contaminants than that of a cultivation-dependent method, there are always limitations (20). Direct PCR analysis can be significantly more expensive, however, the benefits of the direct method clearly outweigh the costs involved. While the extraction of microbes and nucleic acids can be a daunting task (20), the information retrieved is invaluable. The available methodologies are still being refined in pursuit of the goal of accurately isolating and identifying the entire consortia of microbial contaminants. The preliminary results presented here, however, show the potential of using a cultivation-independent methodology to provide a greater understanding of the microbial consortia that contaminate USAF and civilian aviation fuel systems.

5. Conclusions

This study demonstrated the use of a cultivation-independent method as a promising tool to more accurately and comprehensively characterize the microbial contamination in aviation fuel. The previous methodologies dependent on microbial cultivation were shown to be inconclusive, as the literature suggests (15). The direct PCR method used in this study yielded promising results, but the study is not yet complete. The collection of additional military and civilian samples, as well as further improvement of the direct PCR method, should lead to a well-defined, relatively simple, and comprehensive method to qualitatively analyze aviation fuel microbial contaminated samples. The completion of field-based analyses will yield a more accurate picture of the microbial contaminants that exist in aviation fuel. Subsequent studies will include more laboratory-based analyses to understand not only what microbes exist in jet fuel, but also how they create biofilms, etc. and ultimately cause harm to fuel systems.. This approach will create avenues for the development of novel, target specific, and successful mitigation strategies to attack the problem. The eventual goal is to prevent the initial formation of a complex microbial community in aviation fuel systems.

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